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SOYBEANS AND RADISH LEAVES CONTAIN ONLY ONE OF THE SULFONIUM DIASTEREOISOMERS OF *S*-ADENOSYLMETHIONINE

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Key Word Index—*Glycine max*; Leguminosae; soybean; *S*-adenosylmethionine; *S*-adenosylethionine; sulfonium diastereoisomers; liquid chromatography.

Abstract—Using a liquid chromatography method that separates the two sulfonium diastereoisomers of adenosylmethionine, we have found that immature soybeans, soybean callus culture, radish leaves, yeast and rat liver contain only the (*S*)-sulfonium form of *S*-adenosylmethionine. Our findings contradict the suggestion by Stolowitz and Minch that 10–20% of naturally-occurring adenosylmethionine may have the (*R*)-configuration at the sulfonium pole. Absence of the (*R*)-sulfonium isomer of adenosylmethionine in biological materials indicates that the (*R*)-sulfonium form of adenosylmethionine present in commercial adenosylmethionine samples is an artifact of the isolation procedure. Our method of measuring the isomers of adenosylmethionine enabled us to readily determine the rate of racemization and hydrolysis of adenosylmethionine. Our rate constants for racemization (K_r) and hydrolysis (K_h) were 2.4×10^{-6} sec^{-1} and 12.3×10^{-6} sec^{-1}, respectively; values which are noticeably different from those of Wu and co-workers which were obtained with a more complicated method ($K_r = 8 \times 10^{-6}$ sec^{-1}; $K_h = 6 \times 10^{-6}$ sec^{-1}). We believe the absence of the (*R*)-isomer *in vivo* is best explained by stabilization of the (*S*)-isomer as suggested by Wu *et al.* Although the tissues we have analysed contained the (*S*)-sulfonium form of adenosylmethionine exclusively, when ethionine-resistant soybean cell lines were given ethionine, they accumulated both sulfonium diastereoisomers of adenosylethionine.

INTRODUCTION

S-Adenosylmethionine (AdoMet) has a remarkable variety of biochemical functions. It is an allosteric enzyme effector [1], and a precursor in the biosynthesis of spermine, spermidine [2, 3] and ethylene [4]. AdoMet is also the methyl group donor for most biological transmethylation reactions [5], wherein transfer of its methyl group converts AdoMet to the homocysteine analog (AdoHcy). Much of the chemistry and biochemistry of AdoMet derives from the fact that it is a sulfonium compound.

Sulfonium compounds, which have three different substituents on the sulfur atom, such as AdoMet, show stereoisomerism. AdoMet contains five chiral centers besides the sulfonium center. Therefore, when all of the other chiral centers remain the same, there are two sulfonium diastereoisomers of AdoMet. Diastereoisomers have different physical properties and can potentially be resolved by conventional separation techniques. Methylation of *S*-adenosyl-L-homocysteine with iodomethane produces a mixture of the two sulfonium diastereoisomers of AdoMet [6]; however, no method for separation of the two diastereoisomers has been published. In early studies with synthetic AdoMet, approximately 50% of the synthetic AdoMet was utilized by various transmethylation enzymes, and thus it was deduced that only one of the two sulfonium diastereoisomers is recognized by transmethylation enzymes [6, 7]. By comparing the optical rotations of enzymatically-synthesized AdoMet and non-enzymatically-synthesized AdoMet, De La Haba *et al.* [6] determined that enzymatically-formed AdoMet is laevorotatory. Later, on the basis of X-ray diffraction data, Cornforth *et al.* [8] showed the absolute configuration of enzymatically-formed AdoMet at the sulfonium center is (*S*). The (*R*)-sulfonium diastereoisomer of AdoMet has been detected in commercial AdoMet by nuclear magnetic resonance [9] and by enzymatic analysis [10], but it has not previously been established whether the enzymatically inactive isomer in commercial AdoMet is present in the yeast from which commercial AdoMet is isolated, or is an artifact of the AdoMet isolation process. In this paper we report the separation of the two sulfonium diastereoisomers of AdoMet by liquid chromatography, and we report the use of our separation method to show that soybean seeds, soybean callus culture, radish leaves, yeast, and rat liver contain only the (*S*)-sulfonium diastereoisomer of AdoMet.

RESULTS

Other investigators have noted that commercial AdoMet contains impurities [7, 11] but no analyses

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Table 1. Composition of commercial AdoMet preparations

	Sigma* grade I iodide salt Lot 91F-71401	Sigma grade II chloride salt Lot 53F-7010	Sigma grade I $pTSO_3$‡ salt Lot 23F-7190	Calbiochem† iodide salt Lot 610085
	Percent of absorbance units (260 nm)			
AdoMet (*S*-configuration at sulfonium pole)	46.0	69.0	65.1	17.8
AdoMet (*R*-configuration at sulfonium pole)	14.5	9.7	14.5	7.0
AdoHcy	22.4	5.1	6.2	37.6
MTA	11.5	14.3	11.9	37.1
Adenine	2.7	2.0	2.4	1.0

*The Sigma samples were received in dry ice, stored overnight at −15°, dissolved in 0.1 N HCl the next day and analysed.
†The Calbiochem sample was shipped without ice. It was analysed within 24 hr of receipt.
‡*p*-Toluenesulfonic acid.

of AdoMet samples have been published. We have analysed AdoMet samples from Sigma Chemical Company and from Calbiochem-Behring and have found that they contained only 25–80% AdoMet. Other components were identified as AdoHcy, adenine (Ade) and methylthioadenosine (MTA) (Table 1). Twelve to 28% of the AdoMet fraction had the unnatural (*R*)-configuration around the sulfonium pole.

Purified samples of Ade and MTA were quantitatively recovered in our system, even though recoveries of these compounds were less than 60% when they were added to tissue homogenates. Thus we were able to accurately measure Ade and MTA in commercial AdoMet samples. MTA eluted from the strong cation exchanger (SCX) columns in the same fraction as AdoHcy, and its elution time with the amino acid analyser was 32 min (Fig. 1B).

Selective elution from SCX was used to separate AdoHcy, Ade, and MTA from commercial AdoMet (Fig. 1). When AdoHcy, Ade, and MTA were eluted from the SCX column with Solution A (see Experimental), two compounds which were UV-absorbing and ninhydrin-positive, remained bound and were eluted with Solution B (Fig. 1C). On the basis of their chromatographic behavior, UV absorbance spectrum and ninhydrin reactivity, we suspected these two compounds were the two sulfonium diastereoisomers of AdoMet. To test this interpretation, we synthesized the two sulfonium diastereoisomers of AdoMet by methylation of Ado-L-Hcy with iodomethane [6]. Chromatography of the synthetic AdoMet yielded two peaks (Fig. 2C) which coincided with the two peaks from the purified commercial AdoMet. AdoMet enzymatically synthesized with a crude extract from soybean seeds, ATP, and ^{14}C-methionine, appeared as a single peak of radioactivity, coinciding with peak 1 (Fig. 2C) from the synthetic AdoMet sample. This was not surprising, since extracts from soybeans (Fig. 2A), radish leaves, yeast and rat liver, had yielded a UV-absorbing, ninhydrin-positive peak which coincided with peak 1 of the synthetic AdoMet sample.

Evidence that peak 2 in the synthetic and the commercial AdoMet samples was a diastereoisomer of AdoMet was obtained by heating the AdoMet-containing fraction of a soybean sample at 100° for 5 min. This heating diminished peak 1 from its original size, and simultaneously gave rise to a second peak, which absorbed UV light, was ninhydrin positive, and coincided with peak 2 from the synthetic and purified commercial AdoMet samples (Fig. 2B). To verify that peak 2 in the synthetic AdoMet sample was indeed an AdoMet diastereoisomer, rather than an AdoMet decomposition product, we

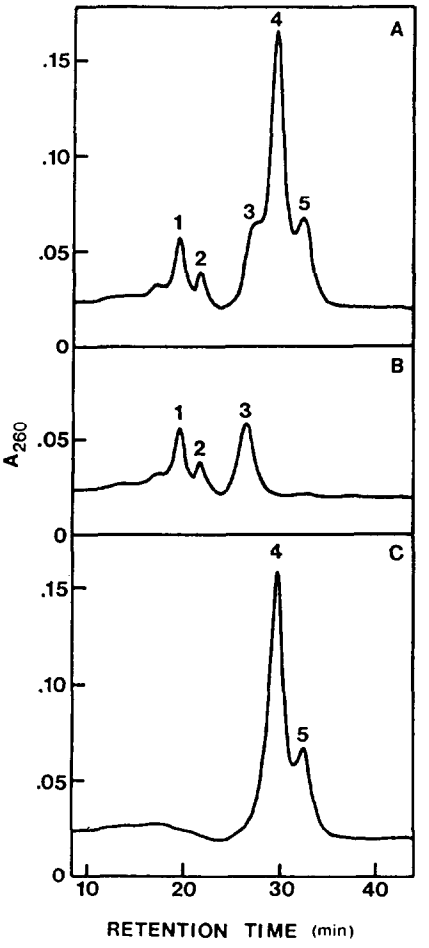

Fig. 1. Chromatograms of commercial AdoMet (Sigma Grade I, column 3, Table 1). (A) Unfractionated AdoMet sample. (B) Solution A eluate from SCX column. (C) Solution B eluate from SCX column. Peaks are as follows: (1) AdoHcy, (2) Ade, (3) MTA, (4) (*S*)-AdoMet, (5) (*R*)-AdoMet.

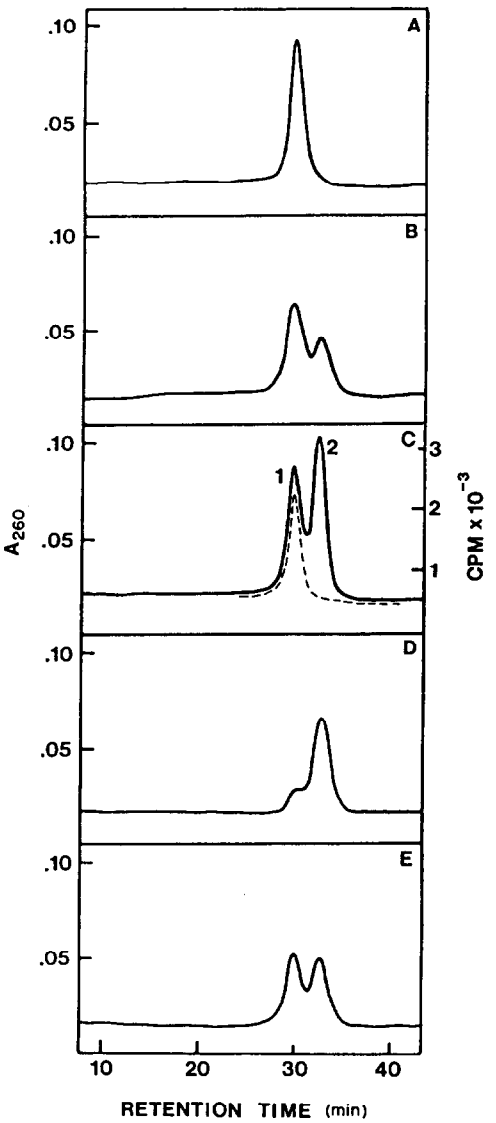

Fig. 2. Chromatograms of diastereoisomers of AdoMet. (A) AdoMet from soybean seeds, obtained by elution with solution B from an SCX column. (B) Solution used in panel A heated at 100° for 5 min. (C) AdoMet synthesized by reaction of iodomethane with AdoHcy (solid line); Enzymatically-synthesized radioactive AdoMet (dotted line). About 20000 cpm of Ado-[^{14}C]Met was added to an aliquot of synthetic AdoMet and chromatographed as usual. Fractions (0.38 ml) were collected and 40 μl aliquots of each fraction were counted in a liquid scintillation counter. (D) Fractions containing peak 2 from a synthetic AdoMet sample were collected as above, combined, diluted with 10 volumes of H_2O and fractionated on a SCX column as usual. The solution B-eluate was chromatographed on the amino acid analyser. (E) Same as (D) except that the solution B-eluate from the SCX column was heated at 100° for 5 min prior to analysis.

chromatographed a sample of synthetic AdoMet with the amino acid analyser and collected peak 2 (Fig. 2D). Heating this fraction at 100° for 5 min increased the material in the peak 1-position as peak 2-diminished (Fig. 2E).

Relative peak heights and elution times were the same for Ado-L-Met and Ado-D-Met synthesized by methylation of AdoHcy (data not shown).

When soybean callus tissue was analysed for AdoMet, the same pattern as for soybean seeds (Fig. 2A) was obtained. This same pattern was seen when the cells were cultured with 0.1 mM L-methionine or 0.4 mM D-methionine. In contrast, when these soybean cells were grown with (D,L)-, (D)-, or (L)-ethionine, the pattern shown in Fig. 3B was found. Peaks 2 and 3 coincide with the two peaks seen when synthetic *S*-adenosylethionine (AdoEth) was chromatographed (Fig. 3A). Peak 2, then, is probably (*S*)-AdoEth, and peak 3 is probably (*R*)-AdoEth. Peak 1 is (*S*)-AdoMet.

The relationship between peak area and amount of AdoMet or AdoHcy standards was linear to at least 25 nmol (data not shown) with both the UV detector and the ninhydrin detector. The lower limits for quantitation of AdoMet and AdoHcy was around 0.5 nmol with the ninhydrin detector and 0.2 nmol with the UV detector. Table 2 shows amounts of AdoMet and AdoHcy found in a variety of biological tissues analysed by our method. The ratio of AdoMet to AdoHcy was considerably larger in plant tissues than in liver and yeast. Both chromatographic detectors gave satisfactory chromatograms of samples from all of the tissues listed, except yeast. There were no problems when the UV-detector was used with yeast extracts, but when the ninhydrin detection system was used the AdoHcy peak was obscured and there was distortion of the baseline around the AdoMet peak.

When tissue samples larger than 3 g (fr. wt) were processed as prescribed, partial loss of AdoHcy was observed. With tissue samples larger than 5 g, similar loss of AdoMet was observed. Four days after elution from the SCX column, a sample of AdoMet stored at 4° had undergone no measurable degradation. After 21 days at 4°, isomerization at the sulfonium pole was detectable, but conversion was less than 5% of the total. There was no

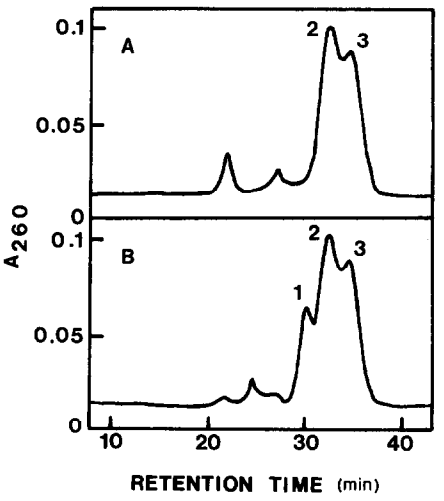

Fig. 3. Chromatograms of diastereoisomers of AdoEth. (A) AdoEth synthesized by the reaction of iodoethane with AdoHcy. (B) Solution B-eluate from SCX column fractionation of an extract of soybean callus tissue that had been grown on 0.2 mM DL-ethionine. Peak 1 is (*S*)-AdoMet; peaks 2 and 3 are the diastereoisomers of AdoEth.

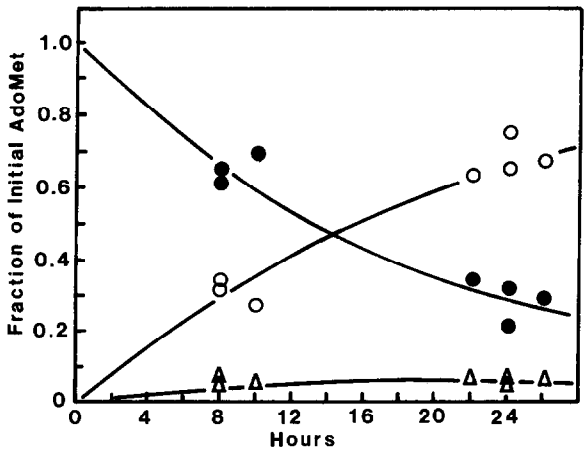

Fig. 4. Kinetics of the degradation of (*S*)-AdoMet. (*S*)-AdoMet was incubated in 30 mM Hepes buffer, pH 7.5, at 37°. At the times indicated, aliquots were chromatographed to separate the diastereoisomers of AdoMet. ●, (*S*)-AdoMet; Δ, (*R*)-AdoMet; ○, MTA (determined by difference).

measurable degradation of an AdoHcy sample which had been stored at 4° for 21 days in Solution A.

Figure 4 shows the amounts of (*S*)-AdoMet and (*R*)-AdoMet remaining when AdoMet was incubated at pH 7.5 and 37° in 30 mM Hepes buffer for various times. The rate constants for racemization (K_r) and hydrolysis (K_h) were determined to be $2.4 \pm 0.4 \times 10^{-6}\,sec^{-1}$ and $12.3 \pm 0.9 \times 10^{-6}\,sec^{-1}$, respectively. These values are to be compared to those of Wu *et al.* [10], who reported K_r to be $8 \times 10^{-6}\,sec^{-1}$ and K_h to be $6 \times 10^{-6}\,sec^{-1}$.

DISCUSSION

Enzymological [10], and NMR studies [9] have indicated that 18–20% of the AdoMet in commercial preparations is in the enzymatically-inactive (*R*)-configuration, rather than the enzymatically-active (*S*)-configuration. Our data (Table 1) from four commercial preparations directly confirm these results. Our chromatographic method also confirmed the finding of Stolowitz and Minch [9] that 60–70% of AdoMet synthesized by methylation of AdoHcy is in the (*R*)-configuration (Fig. 2C). Moreover, Stolowitz and Minch [9] combine this finding with reports [6, 7] that synthetic AdoMet has about half the enzymological activity of AdoMet purified from natural sources, and suggest that 10–20% of AdoMet in natural sources may be in the (*R*)-configuration. However, we did not detect (*R*)-AdoMet in any of the biological tissues listed in Table 2 (see Fig. 2A). We therefore conclude that the (*R*)-AdoMet in commercial preparations is an artifact of isolation.

The rate constant that we determined for the racemization of AdoMet (K_r) is less than a third of that reported by Wu *et al.* [10] ($2.4 \times 10^{-6}\,sec^{-1}$ vs $8 \times 10^{-6}\,sec^{-1}$) under similar conditions. Conversely, our rate constant for hydrolysis (K_h) is twice as large as they found ($12.3 \times 10^{-6}\,sec^{-1}$ as compared to $6 \times 10^{-6}\,sec^{-1}$). Using our values for K_r and K_h, at equilibrium, (*R*)-AdoMet should account for 14% of the total AdoMet as compared to 36% using the rate constants of Wu *et al.*

In either case, since there is no detectable (*R*)-AdoMet in tissue samples, there must be a biological mechanism which prevents accumulation of (*R*)-AdoMet within those cells. Wu *et al.* [10] conclude that the absence of the (*R*)-sulfonium diastereoisomer of AdoMet in tissues is due either to a "binding entity capable of chemical stabilization" or to rapid turnover of AdoMet. We believe the latter suggestion is invalid because the rate of product formation in a first order decay process depends on the size of the population of decaying molecules and not on their age. There are two general possibilities that could account for lack of (*R*)-AdoMet in biological tissues: (a) Inhibition of racemization of (*S*)-AdoMet. This might be through binding to an intracellular component, as suggested by Wu *et al.* [10]. This component could be an AdoMet binding protein such as that reported by Smith [12]. (b) (*R*)-AdoMet is removed enzymatically. For example, it could be hydrolysed or converted back to the (*S*)-isomer.

Table 2. AdoMet and AdoHcy content of various tissues

	AdoMet	AdoHcy
	nmols/g fr. wt	
Immature soybean seed (300–400 mg)	19.7 ± 1.0*	2.2 ± 0.1*
Radish leaf	7.0 ± 0.3*	< 0.6*
Soybean callus	4.1 ± 0.6‡	< 0.6‡
Baker's yeast (stationary phase)	108.0†	29.0†
Rat liver	48.5 ± 1.6*	29.3 ± 3.1*

*Mean value ± SE, $n = 3$.
†Value for a single determination.
‡$n = 11$.

A sample clean-up step for AdoMet and AdoHcy analysis may not be necessary for some biological materials, but in our experience, some type of sample clean-up is required for most plant tissues. The preparation method presented here combines several desirable features. AdoMet and AdoHcy are separated out of the same sample with a single column. The process is fairly rapid, requiring only about 1 hr, and with a vacuum manifold a number of samples can be processed simultaneously. Harsh chemical conditions, such as 6 M HCl [11] and 3 M H_2SO_4 [13, 14] are avoided, and no evaporation step is involved [11]. Since recoveries are quantitative, isotope dilution techniques are not necessary [14]. Finally, AdoMet and AdoHcy are recovered in solutions in which they are stable.

We suggest 3 g of tissue as the limit when using the small SCX columns. The advantage of the SCX column over other packings, such as Dowex 50, which also have benzenesulfonic acid functional groups, is that it has an HPLC-grade silica base, which allows a higher flow rate, and allows samples to be eluted in smaller volumes. The necessary trade-off is a decrease in binding capacity.

EXPERIMENTAL

Sources of materials. Ado-L-Hcy, Ado-D-Hcy, AdoEth and MTA were purchased from Sigma Chemical Co. AdoMet was purchased from both Sigma and Calbiochem-Behring. Bond Elut disposable SCX columns (3 cc) were purchased from Analytichem International (Harbor City, CA).

Instruments. Chromatographic analysis was done with a Beckman model 119CL amino acid analyser. A short column (0.6 cm × 10 cm) of Beckman W-3 resin was used for all analyses. A Gilford-modified Beckman DU spectrophotometer with a flow cell (1 cm path length) and chart recorder served as a UV detector.

Buffers. Buffer 1 contained 0.12 M Na_3 citrate, 0.58 M NaCl, 0.23 M KCl, pH 4.0. Buffer 2 was the same except that the pH was 7.0. HCl was used to adjust pH.

Chromatography. Buffer 1 was pumped at 44 ml/hr for 5 min, followed by buffer 2 at the same rate for 25 min. The column temperature was 50° for the first 3 min of each run and then 65° for the remainder of each run. The column was regenerated with 11 ml of 0.2 M NaOH, 10 mM EDTA (15 min), followed by 11 ml of buffer 1 (15 min). The ninhydrin flow rate was 22 ml/hr. When the UV detector was used, a ninhydrin reaction coil was inserted into the line between the column and the flow cell to prevent bubble formation due to pressure drop, and to make the elution times comparable for both detectors. Peak areas were determined by electronic integration or by the height times width at half-height.

Sample preparation. Immature soybean seeds (300–400 mg fr. wt per seed), soybean callus culture, radish leaves and rat liver (*ca* 3 g each), were homogenized with 3 vols of 0.2 M $HClO_4$ in a Servall Omnimixer.

Yeast cells (grown to stationary phase from commercial baker's yeast on medium composed of 10 g/l of glucose and 7 g/l of Difco yeast nitrogen base) were ruptured by sonication. Yeast (3 g) was extracted with 10 ml of 0.2 M perchloric acid. Insoluble material was pelleted by centrifugation at 7500 *g* for 10 min and then washed twice with 2.5 ml of 0.2 M $HClO_4$. The crude extracts (15 ml) were diluted 4-fold with water and passed through Bond Elut SCX columns, which had been pretreated with 5 ml MeOH followed by 5 ml H_2O. Mild suction was used to give flow rates of about 6 ml/min. The column was washed (in this sequence) with 9 ml of 0.5 M HCl; 6 ml H_2O; 6 ml of 80% EtOH; and finally with 3 ml of 50 mM triethanolamine-acetate (pH 5.0) in 25% EtOH [Solution A]. AdoHcy was then eluted with 6 ml of Solution A. Next, AdoMet was eluted with 4 ml of 1.5 M KCl, 0.5 M HCl, 15% EtOH [Solution B]. Elution of AdoHcy and AdoMet was done without suction, so that the flow rate was about 1 ml min^{-1}. The Bond Elut SCX columns were discarded after one use. Aliquots (up to 1.0 ml) of the SCX column eluates were loaded directly into the amino acid analyser.

Purification of commercial AdoMet. Commercial AdoMet (25 mg), dissolved in 5 ml of water was applied to a carboxymethylcellulose-H^+ column (0.9 × 5.0 cm). The column was washed with 10 ml H_2O and 10 ml of 80% EtOH. The AdoMet was eluted in 10 ml of 0.1 M HCl and stored at 4°. This procedure freed the AdoMet solution from contaminating MTA, Ade, and AdoHcy, so that on the basis of its absorbance at 260 nm, it could be used as a standard for calibration purposes. We found no degradation of AdoMet stored in 0.1 M HCl at 4° for 2 months.

Synthesis of AdoMet and AdoEth from AdoHcy. AdoMet was synthesized from AdoHcy by a modification of the procedure of De La Haba *et al.* [6]. Ado-L-Hcy (12 mg) was dissolved in formic acid (200 μl) in a 0.5 ml vial. Iodomethane (125 μl) was added to the soln. The vial was capped tightly and left in darkness at room temperature for 4 days. The excess iodomethane and formic acid were then removed by evaporation *in vacuo*. In a similar way Ado-D-Met was prepared from Ado-D-Hcy and AdoEth was prepared by substituting iodoethane for iodomethane.

Enzymatic synthesis of AdoMet. Fresh immature soybean seeds were ground in liquid nitrogen, and proteins were extracted with 200 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM mercaptobenzothiazole, 5 mM DTE. Insoluble material was pelleted by centrifugation at 10 000 *g* for 15 min. Ammonium sulphate was added to 80% of saturation and after 20 min centrifuged as before. Low molecular weight compounds were removed by passage through a Sephadex G-25 column (2.5 cm × 25 cm) in 50 mM Tris–HCl (pH 7.5), 1 mM DTE. The protein-containing fraction from the G-25 column was mixed with an equal volume of 50% glycerol (v/v) and stored at −80°. The reaction mixture for synthesis of ^{14}C-AdoMet had a total volume of 200 μl, and contained the following: 100 μl of enzyme extract (0.67 mg protein), 30 μmol of Tris–HCl (pH 7.6), 20 μmol of KCl, 4 μmol of $MgCl_2$, 2 μmol of ATP and 0.1 μCi of ^{14}C-methionine (5 mCi/mmol). Incubation was for 2 hr at 30°. Unlabeled AdoMet was synthesized in a final volume of 2 ml with 5 μmol of methionine. Incubation was for 7 hr in the presence of chloroform. After the specified times the reaction mixtures were frozen.

Rate of racemization of AdoMet. Unlabeled AdoMet from the preparation described above was isolated after adding 5 ml EtOH and centrifuging at 4° for 20 min at 7500 *g*. The supernatant was applied to a 0.9 by 5 cm column of carboxymethylcellulose. After washing with 10 ml of water, AdoMet was eluted with 6 ml of 0.1 M HCl. Hepes buffer was added to a final concentration of 30 mM and the pH was adjusted to 7.5 with KOH. This soln was incubated at 37°. At the indicated times aliquots were removed, acidified with the addition of trichloroacetic acid to 0.3 M and the diastereoisomers of AdoMet separated as described above. The rate constants for racemization and hydrolysis were calculated as described by Wu *et al.* [10].

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